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Proposed Biological Testing Methods for the United States Incineration-at-Sea Research Program

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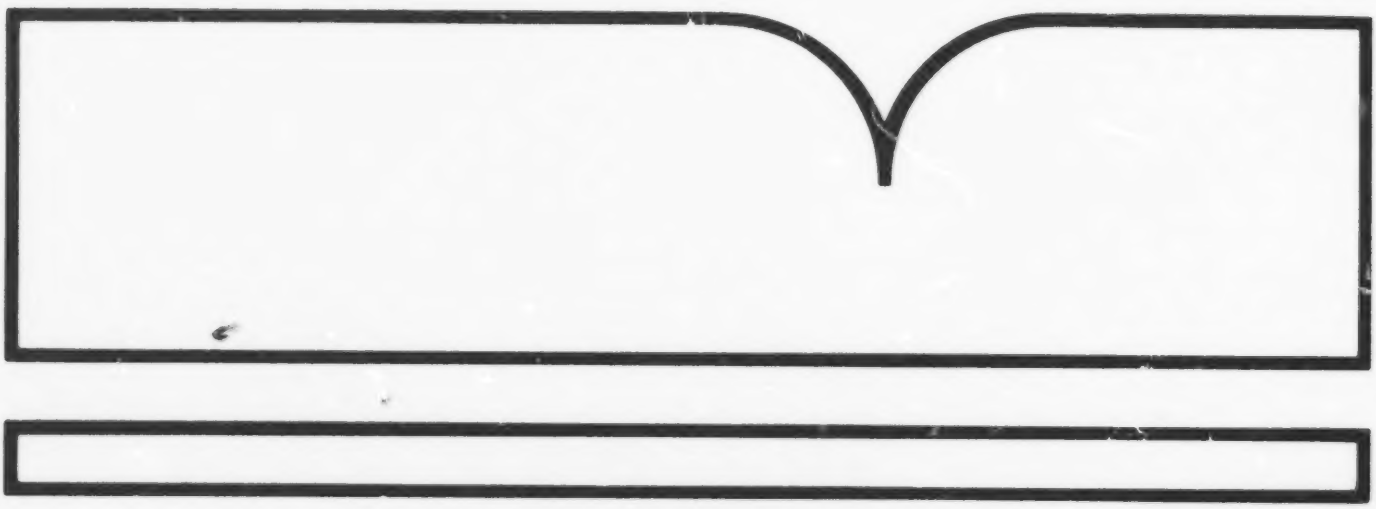

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16. ABSTRACT

As part of the United States Environmental Protection Agency's (EPA) Incineration-at-Sea research programme, a suite of toxicity tests has been selected for assessing the toxicity of incinerator emissions generated during the combustion of chlorinated wastes. The test organisms for the five short-term chronic tests are the inland silverside, *Menidia beryllina*, the mysid *Mysidopsis bahia*, the red macroalga *Champia parvula*, the polychaete *Dinophilus gyrociliatus*, and gametes from the sea urchin *Arbacia punctulata*. The durations of individual tests range from 2 h to 7 days.

The endpoints include survival, growth and reproductive effects. To date there have been three land-based trial burns to evaluate the sampling and toxicity testing methods. The results have demonstrated that the proposed methodologies can be used to test the toxicity of gaseous emissions, and that there appears to be no significant toxicity associated with the combustion products of a carrier fuel oil.

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Proposed Biological Testing Methods for the United States Incineration-at-Sea Research Program

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As part of the United States Environmental Protection Agency's (EPA) Incineration-at-Sea research programme, a suite of toxicity tests has been selected for assessing the toxicity of incinerator emissions generated during the combustion of chlorinated wastes. The test organisms for the five short-term chronic tests are the inland silverside, *Menidia beryllina*, the mysid *Mysidopsis bahia*, the red macroalga *Champia parvula*, the polychaete *Dinophilus gyrociliatus*, and gametes from the sea urchin *Arbacia punctulata*. The durations of individual tests range from 2 h to 7 days.

The endpoints include survival, growth and reproductive effects. To date there have been three land-based trial burns to evaluate the sampling and toxicity testing methods. The results have demonstrated that the proposed methodologies can be used to test the toxicity of gaseous emissions, and that there appears to be no significant toxicity associated with the combustion products of a carrier fuel oil.

At-sea incineration was first used for hazardous waste disposal nearly two decades ago. Since that time, European countries have routinely burned wastes aboard incinerator ships in the North Sea.

Since 1972 the United States Environmental Protection Agency (EPA) has conducted several research burns in the Pacific Ocean and the Gulf of Mexico (Wastler *et al.*, 1975; Clausen *et al.*, 1977; Ackerman *et al.*, 1978, 1983). EPA has developed a research plan (US EPA, 1985) for conducting future at-sea research burns, which, unlike previous burns which focused primarily on documenting destruction efficiency, will include on-board toxicity testing of stack emissions, in addition to numerous other biological and chemical analyses.

This paper briefly describes the biological tests that have been selected by the EPA's Incineration-at-Sea research programme for determining the toxicity of gaseous emissions. To date, three land-based incineration studies have been conducted to evaluate the performance of the toxicity tests and sampling methods. Toxicity data from these test burns are discussed, but the data are considered preliminary and no attempts can yet be made to extrapolate to field situations.

Proposed Methods

A suite of five toxicity tests has been chosen for use by the Incineration-at-Sea research programme. They are classified by EPA as short-term chronic tests, using the inland silverside, *Menidia beryllina*, the mysid *Mysidopsis bahia*, the red macroalga *Champia parvula*, the polychaete *Dinophilus gyrociliatus*, and gametes from the sea urchin *Arbacia punctulata* as test organisms. The durations of the tests range from 2 h to 7 days. The endpoints include growth, survival and reproductive effects.

Criteria used to evaluate the suitability of the above tests include:

- adaptability of the protocol to alternative bioassay system designs such as those used for on-site testing in mobile laboratories;
- the year-round availability of the test species and appropriate life stages;
- bioassay endpoints that can be obtained relatively quickly (7 days or less) and are readily interpretable in terms of potential deleterious marine population or community responses;

- ease with which the test organisms can be cultured in the laboratory and transported to a mobile laboratory site;
- high sensitivity of the test species to the types of toxicants expected in the emissions of hazardous waste incineration; and
- availability of data bases attesting to the reliability and reproducibility of the protocol.

With the exception of the *Dinophilus* test, all tests were developed or modified from existing methodologies at the EPA's Environmental Research Laboratory in Narragansett, Rhode Island (ERL-N). They were developed for the Marine Complex Effluent Toxicity Testing Program to test industrial and municipal effluents discharged into marine and estuarine environments (Horning *et al.*, 1987). The *Dinophilus* test was developed at Battelle Ocean Sciences in Duxbury, Massachusetts (Carr *et al.*, 1986).

All tests are conducted in an EPA-designed mobile testing laboratory which can be placed on board the incinerator ship (Fig. 1). This laboratory is a modified marine shipping container equipped with temperature controlled water baths, hot and cold fresh water, deionized water, and all equipment and supplies needed to perform the five biological tests.

Sampling methods

The biological assays were designed to test the toxicity of effluents or compounds in sea water, it is thus necessary to effectively incorporate the constituents of the stack gas into an aqueous medium for testing. To accomplish this transfer, the Marine Incineration Biological Assessment Sampler (MIBAS) system was developed (Werme *et al.*, 1988; Fig. 2). Stack gases are cooled and then bubbled through sea water contained in a series of glass impingers for 3 h. Gases then pass through XAD-2 resin and two additional impingers for clean-up. The XAD-2 resin is chemically analysed and the last two impingers protect the pump from corrosion. The resulting concentrated solution in the first four impingers, along with a sea water rinse of the first half of the system (from the quartz liner in the probe through the last sea water impinger, inclusive), constitutes the primary solution to be diluted for testing (for a more thorough description of the MIBAS see Werme *et al.*, 1988). Fresh samples are collected daily throughout the test period.

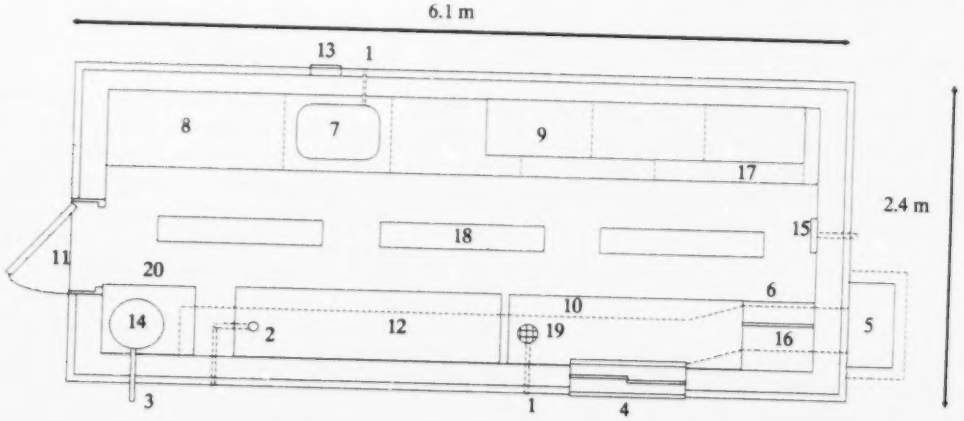
Dilutions of the MIBAS sample are calculated to span the range of concentrations in which effects are expected.

Since HCl is produced during the combustion of chlorinated compounds, the pH of the concentrated MIBAS sample is outside of the tolerance limits of the test organisms (during preliminary tests pH ranged from 0 to 1). Therefore, the pH and salinity of the sample must be adjusted to 8.0 and 30‰, respectively, before testing. Sodium hydroxide and deionized water are used for these adjustments. This neutralization process was deemed acceptable because HCl deposited on the sea surface during an at-sea burn would be rapidly neutralized by the ocean's natural buffering system.

Serial dilution to test concentrations is performed with 30‰ sea water after water quality adjustments are completed. Dilution calculations are based on the volume of the original unadjusted stock. Adjustments and dilutions are performed immediately upon completion of sample collection, and renewals are completed within 4 h.

Toxicity Tests

Menidia beryllina. The fish growth/survival test is a 7 day assay conducted under static renewal conditions (test solutions are renewed daily). Fifteen 7 day old,



1. 3/4" Drain through sidewall w/ garden hose type fitting
2. 1 1/2" Drain through sidewall for water baths
3. 3/4" fresh water supply line w/ garden hose type fitting
4. Window – escape type
5. Heating/air conditioning unit
6. Refrigerator
7. S. S. sink
8. Formica cabinet – sperm cell test area
9. Hanging cabinet
10. Kneehole desk w/ built-in light table
11. Exit door
12. 3-tiered water baths with light control system
13. 8" Exhaust fan
14. Hot water heater, 36 gal
15. Breaker panel
16. Macroalga testing area
17. Formica cabinet w/ 5 drawers
18. Fluorescent lights
19. 4" Floor drain
20. Formica cabinet

Fig. 1 Description of the mobile biological testing laboratory.

laboratory-reared individuals are used per replicate, with three replicates per treatment. Treatments consist of dilutions of the pH- and salinity-adjusted MIBAS sample (10, 3.2, 1.0, 0.32, and 0.1%), a site water control using clean sea water collected in advance from the incineration site (and diluted to 30‰ with DI), and a reference control using reconstituted Narragansett Bay hypersaline brine. Site water is used for all dilutions of the MIBAS samples. Fresh MIBAS samples are obtained daily for renewals.

The test chambers are modified glass Norberg–Mount chambers (rectangular chambers with a sump area for solution renewals; Norberg & Mount, 1985) containing approximately 1 l. of test solution. They are sufficiently stable so that they should not be disturbed by the ship's motion. The chambers are not aerated during the study.

Test animals are randomly distributed into test chambers which are placed in a random array in a 25°C water bath. Temperature, salinity and dissolved oxygen are measured daily just prior to solution renewal. A light/dark cycle of 14/10 h is maintained. Dead animals are removed, recorded and discarded daily. Animals are fed *Artemia* sp. nauplii daily after solution renewal, with care being taken to assure a uniform amount of food per replicate. During solution renewals, excess *Artemia* and debris are removed.

On day 7 (the last day of the test) surviving fish are transferred by replicate to vials containing 4% buffered formalin. Upon returning to the laboratory these fish are dried and weighed. The endpoints for this test are growth (final weight differences between control and experimental animals) and percent survival. All data are statistically analysed by Analysis of Variance (ANOVA) and Duncan's Multiple Range test. Survival data are arcsine root transformed prior to analysis (Horning *et al.*, 1987).

Mysidopsis bahia. The mysid test is a 7 day static renewal assay using the same treatments described above. Test chambers are 355 ml disposable polycarbonate cups (pre-soaked in sea water) containing approximately 150 ml of test solution. There are eight replicates per treatment with five randomly assigned animals per replicate.

The test is initiated with 7 day old laboratory-reared individuals. Test chambers are randomly arranged in racks in a 26°C water bath. Water quality measurements, solution renewals and feeding (*Artemia* sp. nauplii) are performed daily. The test chambers are not aerated during the study.

On day 7, surviving mysids are counted and removed for observation under a dissecting microscope. Numbers of immature animals, males, females with eggs, and females without eggs are recorded to estimate maturity and reproductive potential. Animals are observed live because they turn opaque upon preservation, thereby making the microscopic observations impossible. All individuals are then dried and weighed. Growth (as dry weight), percent survival, and percent females bearing eggs are statistically analysed by ANOVA and Duncan's Multiple Range test. Survival and egg production data are arcsine root transformed prior to analysis (Horning *et al.*, 1987).

Champia parvula. The red macroalga test consists of a static 2 day exposure (no solution renewal) followed by a 5–7 day recovery period. Test concentrations are identical to those described above, except the reconstituted Narragansett Bay hypersaline brine is replaced with autoclaved Narragansett Bay sea water. There are three replicates per treatment with five plants per replicate. The exposure chambers are polypropylene specimen cups containing 80 ml of test solution plus nutrients.

To initiate a test, five female plant tips (*ca.* 1 cm) and one larger male plant tip are randomly placed in each exposure cup. The cups are covered and placed in random order on a shaker table at *ca.* 75 rpm. They are maintained at a temperature of 20°C, a light/dark cycle of 16/8 h and are not aerated. At the end of the exposure period, the male tips are discarded and the female tips placed in unaerated polystyrene tissue culture flasks containing clean site water. Flasks are returned to the shaker table for a 5–7 day 'recovery period'.

At the end of the recovery period, the plants are

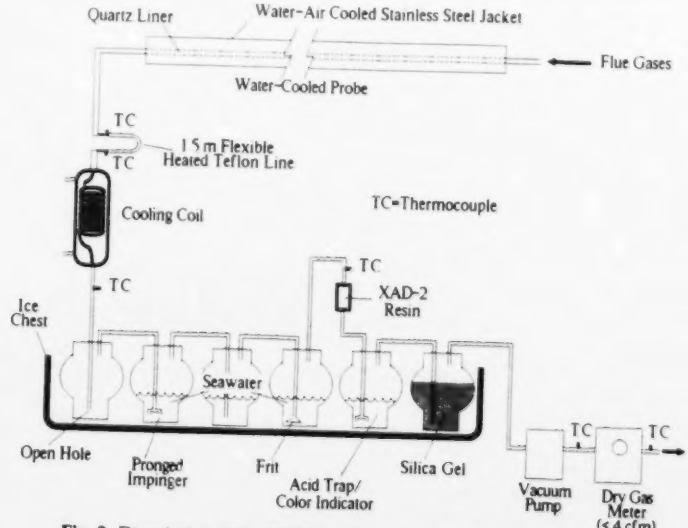


Fig. 2 Description of the MIBAS system (from Redford *et al.*, 1988b).

observed microscopically for cystocarp production. Cystocarps are evidence of sexual reproduction and appear as bumps on the parent plant. ANOVA and Dunnett's test are used to compare the number of cystocarps per plant across treatments (Horning *et al.*, 1987).

Dinophilus gyrociliatus. The polychaete test is a 7 day life-cycle static renewal assay. A minimum of four 1–2 day old juveniles are exposed per replicate. There are five replicates per treatment. Treatments consist of dilutions of the adjusted MIBAS sample (8.3, 4.2, 2.1, 1.0, and 0.5 percent) plus a site water control. Unlike the tests described above, this test is conducted at a salinity of 25‰.

To initiate a test, individuals are randomly placed in glass Stender dishes containing 10 ml of test solution. These chambers are maintained at 20°C and a light/dark cycle of 16/8 h. Due to the limited volume of test solution, dissolved oxygen and salinity are monitored on fresh and 24 h old composite samples for each treatment on days 1 and 6 only. Temperature of the test compartment is monitored continuously with a temperature probe.

Daily observations are made of the number of surviving individuals, number of eggs laid, number of eggs per capsule and the number of juveniles released. Animals are fed once at the start of the test with two drops of a 0.5% (w/v) suspension of 105 µm sieved spinach.

Upon termination of the test on day 7, the above observations are made and the number of eggs visible within the coelom of each worm is also recorded. The endpoints for this test are survival and reproductive success. Four and seven day LC_{50}s are determined by the Spearman-Karber method with Abbott's correction (Hamilton *et al.*, 1977). Reproductive success (the total number of eggs produced per surviving female) is statistically analysed by ANOVA and Duncan's multiple range test (Carr *et al.*, 1986).

Arbacia punctulata sperm cell test. The sea urchin sperm cell test is a very rapid test using egg fertilization as an indicator of sperm viability. The duration of the test is approximately 1.5 h. There are four replicates per treatment. Treatments consist of serial dilutions of the adjusted MIBAS sample and both a site water and reconstituted Narragansett Bay hypersaline brine control. This test is conducted at a salinity of 30‰ and a temperature of 20°C.

To initiate a test, sperm is obtained from four mature males by electrical stimulation. Fifty µl of diluted sperm from one animal is used per chamber for each replicate. Test chambers are glass scintillation vials containing 5 ml of test solution. Vials containing sperm are incubated for 1 h at 20°C. One ml of a diluted egg suspension (also obtained via electrical stimulation) is then added to each vial. All vials are incubated at 20°C for an additional 20 min. and then preserved by adding two ml of 10% buffered formalin. The eggs are then observed under a microscope for the presence of a fertilization membrane, indicating that fertilization has occurred.

The endpoint in this test is percent fertilization per treatment relative to the control. An EC_{50} is determined by the Spearman-Karber method with Abbott's correction (Horning *et al.*, 1987; Hamilton *et al.*, 1977).

Preliminary Verification and Discussion

To date, there have been three land-based test burns within this programme. The purpose of these studies has been to identify and resolve potential problems with the methods and the mobile laboratory prior to an actual at-sea incineration study.

TABLE 1

Results of the biological testing performed on incinerator emissions entrained in sea water during the combustion of uncontaminated fuel oil. Testing was performed at a land-based incinerator in Columbus, Ohio in 1985. 'Sea water' is 30‰ Narragansett Bay sea water. The diluent for the *Champia* test was sea water. Reconstituted brine was used as the diluent for all other tests.

Test	Treatment			
	Sea Water Control	Reconstituted Brine Control	2% MIBAS	2% MIBAS w/HCl
Menidia beryllina				
% survival	91	91	84	91
mean dry weight (mg) ± SD	1.00 ±0.05	1.09 ±0.10	1.13 ±0.05	1.08 ±0.10
Mysidopsis bahia				
% survival	98	92	90	96
mean dry weight (mg) ± SD	0.217 ±0.07	0.216 ±0.06	0.263* ±0.07	0.243 ±0.07
mean % gravid females ± SD	†	†	†	†
Champia parvula				
No. cystocarps/plant ± SD	25 ± 9	15 ± 4	15 ± 6	18 ± 4
Arbacia punctulata				
% fertilization (tested daily)	75.5–95.0	68.6–87.6	70.3–91.4	70.9–94.0‡
Dinophilus gyrociliatus				
% survival (7 d)	100	100	100	100
mean No. eggs/female (day 7)	4.3	2.5§	5.2*	2.5

*Significantly different from control (alpha = 0.05).
†Inconclusive results. Delay in maturation due to low temperatures.
‡1 of 6 samples tested significantly different from control (alpha = 0.05).
§Significantly different from Narragansett sea water control (alpha = 0.05).

During the first study, uncontaminated fuel oil was burned. To simulate conditions encountered during the incineration of a chlorinated waste, the MIBAS sample constituting one of the treatments was spiked with HCl and then pH adjusted. No site water was collected for this study. With the exception of the *Champia* test, reconstituted Narragansett brine was used as the diluent for the MIBAS sample. Narragansett Bay sea water was used for the sea water control and for diluting the MIBAS sample for the *Champia* test. Unlike in subsequent testing, only one concentration, 2% MIBAS, was tested.

The results of this test are shown in Table 1. With the exception of one of six sperm cell tests conducted, no toxicity was demonstrated by any of the tests.

Fuel oil containing 30% PCB was incinerated during the second study. During this study the *Champia* test was performed on three different daily samples, the sperm cell test was conducted daily, and all other tests were performed once with daily renewals. Because of the high sensitivity of the test, the sperm cell test was conducted at a lower range of concentrations than the other toxicity tests.

Toxicity associated with the emissions was measured by all five tests (Table 2). The NOEC (No Observable Effect Concentration—highest concentration tested producing no significant effect on any endpoint) ranged from 0.06% for the sperm cell test to 1.0% MIBAS for the fish and mysid tests. For *Champia* and *Dinophilus*, the lowest concentrations tested produced a significant effect, therefore NOECs could not be calculated.

Several problems with the mobile laboratory and the methodologies were identified. Lighting problems resulted in the failure of the *Champia* test conducted in the field. The *Champia* data in Table 2 were generated at ERL–N using archived samples. Subsequent modifications to the lighting system has eliminated this problem.

A 48 h delay in returning the live mysids to ERL–N for egg production observations forced a modification in the interpretation of the mysid reproduction test. The delay apparently allowed for the development of eggs after the termination of the test. During this time, all animals were kept in uncontaminated sea water and were therefore no longer stressed by the emissions. The majority of experimental females contained eggs in the oviducts alone, whereas control females contained eggs in the oviducts as well as more developed eggs in the brood sac. Eggs found in the brood sacs are older than those in the oviducts, and therefore would more likely have been produced during the test. To compensate for the delay, only those females with eggs in the brood sac were recorded as gravid. During an at-sea study, observations of egg production would be performed on-board the ship immediately upon termination of the test.

During the third study, a mixture of carbon tetrachloride and chlorobenzene was burned. Again toxicity was measured by all five tests. The data have not yet been officially released by the Agency; however, effect concentrations were very similar to those generated during the PCB burn and the order of species sensitivity was the same. The problems described above were not encountered during the third burn.

Assuming a maximum calculated environmental exposure of 0.02% MIBAS (Redford *et al.*, 1988) interpretation of the data might suggest no potential impact.

The unusually high variability in this endpoint may also have been an artifact of the delay. However, the sampling efficiency of the MIBAS is still unknown. Pre-

TABLE 2

Results of the biological testing performed on incinerator emissions entrained in sea water during the combustion of fuel oil contaminated with PCBs. Testing was performed at a land-based incinerator in Jefferson, Arkansas in 1986. All dilutions were performed with site water. (Data also published in Boehm *et al.*, 1988.)

Test	Treatment						
	Brine Control	Site Control	0.1% MIBAS	0.32% MIBAS	1.0% MIBAS	3.2% MIBAS	10% MIBAS
Menidia beryllina							
% survival	91	95	95	96	93	76*	4*
mean dry weight (mg) ± SD	0.495 ±0.012	0.475 ±0.024	0.524 ±0.055	0.521 ±0.029	0.487 ±0.090	0.428 ±0.005	0.216†
Mysidopsis bahia							
% survival	—‡	85	80	—‡	83	35*	0*
mean dry weight (mg) ± SD	—‡	0.33 ±0.04	0.36 ±0.08	—‡	0.29 ±0.06	0.32 ±0.03	—
mean % gravid females ± SD§	—‡	30 ±34	31 ±34	—‡	14 ±35	0	—
Champia parvula							
No. cystocarps/plant (3 tests run)	15‖	12	0.1–3.2*	0–0.7*	0*	0*	0*
Arbacia punctulata							
% fertilization (tested daily)		EC_{50} = 0.089–0.821			NOEC = 0.062–0.125		
Dinophilus gyrociliatus							
survival		7-day LC_{50} = 1.4			NOEC = 1.0		
egg production		NOEC = <0.5					

*Significantly different from control (alpha = 0.05).
†Significantly different from control (alpha = 0.05), however n = 2.
‡Concentration not tested due to inadequate number of animals.
§Due to a delay in observing females for the presence of eggs, 'gravid' is defined for this test only as presence of eggs in brood sac, not oviducts.
‖Narragansett Bay sea water in place of reconstituted brine.

liminary spike-recovery experiments have not produced quality-assured reproducible data, and results from subsequent studies are not yet available. Until these data are evaluated, the toxicity test concentrations cannot be related to potential field exposures. As a result, any predictions of potential environmental effects (or lack of an effect) based on these data would be inappropriate.

Research conducted during the three land-based test burns demonstrate that the proposed biological testing methodologies can be successfully used to measure the toxicity of gaseous emissions generated during the incineration of chlorinated wastes. The lack of measurable toxicity associated with the fuel oil burn, and the observed toxicity produced by the combustion of chlorinated compounds (in which fuel oil was used as a carrier) suggest that the tests are sensitive to the types of compounds expected to be produced during the incineration of hazardous wastes at-sea, and that any toxicity observed would probably be due to the combustion of the waste and not the carrier fuel oil.

The mobile biological testing laboratory was evaluated and it was concluded that this facility can be successfully used for conducting on-board toxicity tests during ocean incineration cruises.

With verification of the biological tests completed, once the efficiency of the MIBAS sampler has been fully documented the proposed testing methods could be employed to measure the toxicity of emissions generated during actual waste incineration.

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